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13. ABSTRACT *(Maximum 200 Words)*

Our previous work demonstrated that prostate cancers differ from benign prostatic epithelium in their expression of oncogenic members of the pp32 gene family. Whereas benign prostatic epithelium solely expresses pp32, a tumor suppressor, prostate cancers express pp32r1 and pp32r2, which are oncogenic. The purpose of the study is to confirm and extend these preliminary results, to develop practical means to assay pp32 gene family members in clinical samples, and to determine the clinical significance of their presence. The approved proposal encompassed four broad tasks: [1] characterization of the pp32 expression phenotype of a larger sample of 40 prostatic adenocarcinomas; [2] development of a practical molecular pathology assay for altered pp32 transcripts; [3] adaptation of the assay to paraffin-embedded tissue; and [4] preliminary determination of the clinical utility of pp32r1 and pp32r2 expression in prostatic adenocarcinoma. Unanticipated difficulties were encountered with the assay developed during the previous funding period. During the present funding period, a competitive quantitative PCR assay compatible with RNA from paraffin sections was developed. In the period beyond the funding period, these tools shall finally be applied to completion of Task 1 and Task 4.

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INTRODUCTION

Since this study was first proposed, parallel fundamental work in the biology of pp32 by our laboratory and others has brought pp32 to central prominence as a regulator of histone acetylation, and through this function, gene expression in a molecular sense; in a biologic sense, pp32 plays a major role in the control of cell differentiation. In turn, this knowledge provides a more compelling, if more complex rationale for the careful analysis of the pp32 gene family in cancer than existed at the outset of this study. Most importantly, it credibly points to a potential role of pp32 as a target of therapeutic intervention. To summarize a complex literature briefly, pp32 frequently co-isolates with the SET oncoprotein. The pp32-SET complex are key components of a potent inhibitor of histone acetyl transferases (1); acetylation of histones facilitates gene expression by modifying chromatin structure and making genes more accessible to transcription factors and other machinery required for gene expression. The domain of pp32 required for inhibition of histone acetylation is the same region that we identified as being required for tumor suppression (2,3). We have recently shown that antisense inhibition of pp32 drives cells to differentiate and cease proliferation; antisense inhibition of pp32 is accompanied by decreased expression of the proliferative marker PCNA, modulation of histone acetylation, expression and elaboration of autocrine differentiation factors, and acquisition of differentiation markers (4). These studies were initially carried out in the TSU-Pr1 cell line, originally thought to be a prostatic adenocarcinoma but now considered to be more consistent with a human bladder carcinoma. Confirmation was obtained in human neoplastic hematopoietic cell lines. The studies are now being extended to human prostate cancer.

The approved proposal encompassed four technical objectives: [1] characterization of the pp32 expression phenotype of a larger sample of 40 prostatic adenocarcinomas; [2] development of a practical molecular pathology assay for altered pp32 transcripts; [3] adaptation of the assay to paraffin-embedded tissue; and [4] preliminary determination of the clinical utility of pp32r1 and pp32r2 expression in prostatic adenocarcinoma.

BODY

Task 1. This approved task involves characterization of abnormal pp32 transcripts in frozen samples of human prostatic adenocarcinoma compared to paired normal prostate controls. 40 pairs of prostatic adenocarcinoma and normal prostate are to be analyzed to determine the range and frequency of occurrence of pp32 gene family-related sequences in prostatic adenocarcinoma.

Progress: In the previous report, we noted that work on this task had been deferred pending development of the assay outlined in Task 2. At that time, there was considerable optimism regarding the assay under development in Task 2, however that optimism was ultimately unjustified. Significant quantitative differences exist in the levels of expression of pp32, pp32r1,

and pp32r2. The assay described last year was suitable for pp32, but not for pp32r1 and pp32r2, which are now known to be expressed at substantially lower levels. The assays for these molecules were complicated by significantly higher backgrounds than tolerable in assays applied to clinical material. Therefore, the present reporting period was largely devoted to further assay development and refinement.

Task 2. This approved task aims at development of a practical molecular pathology assay to distinguish individual members of the pp32 gene family. Briefly, this task involves selection and optimization of PCR primer sets for efficient amplification of altered regions of pp32. The original objective was to select and optimize restriction enzyme cleavages to distinguish among normal pp32 and the various altered forms of pp32. This involved standardization of the assay using defined mixtures of plasmid DNA to determine sensitivity and specificity under optimized PCR conditions and comparison of assay performance on known samples of RNA from frozen tissue.

Progress:

Table I. Quantitation of pp32 Gene Family Members in Prostate Cancer Cell Lines

Cell line	pp32	pp32r1	pp32r2
DU145	590160	3	50
LNCaP	552039	269	99
PC-3	191412	161	24
TSUPr-1	851079	2	49

The data are expressed as copies per 100 ng total RNA.

Table I illustrates the unanticipated and striking differences in expression levels of pp32, pp32r1, and pp32r2 that rendered the assay described in the previous report unreliable in estimating quantitative differences among the pp32 gene family members. In its place, in conjunction with an ongoing DAMD-funded breast cancer project, a quantitative assay was developed for use in both projects. Briefly, a competitor approach was developed to quantitate the expression of the three pp32-related genes. We also intended to co-reverse transcribe and then co-amplify the competitor and the endogenous sequences with a single primer set specific for each of the three pp32-related transcripts. Co-amplification of the endogenous sequence and the competitor in the same tube with one primer set eliminated the possibility of variations in amplification efficiency between the endogenous sequence and the competitor. We first PCR synthesized

three separate competitor DNA sequences with forward and reverse primer sequences specific for pp32, pp32r1 or pp32r2. The sequence internal to the primer sites was different from any pp32-related sequence although the percentage G+C and A+T was approximately the same as each of the pp32 related genes. The amplicon size from competitor was 280bp which could be easily distinguished from the amplicons generated by the endogenous genes (pp32, pp32r2 - 195bp; pp32r1 - 185bp) on 2% agarose gel electrophoresis. The competitor DNA was subcloned into an expression vector, pCR II TOPO (Invitrogen, Carlsbad, CA). Sense strand mRNA was generated from each of the three competitors utilizing T7 or Sp6 RNA polymerases and the MegaScript in vitro transcription kit (Ambion Inc., Austin, TX). The in vitro transcribed RNA was DNase treated to eliminate template DNA and absence of DNA was confirmed by PCR-only reactions without reverse transcription.

Quantitative RT-PCR was performed using the One step RT-PCR kit (Qiagen). Three tubes each with a constant amount of RNA but with varying amounts of competitor RNA were used to analyze the quantitative expression of the three pp32-related genes from each sample. The amounts of input RNA, copies of the competitor RNA, and the number of PCR cycles used are shown in the tables accompanying each graph. The RT step was carried out at 52° for 45 minutes followed by inactivation of the reverse transcription for 15 minutes at 95°. This step also simultaneously activated the HotStar Taq polymerase. Each PCR cycle consisted of denaturation at 92° for 30 sec, primer annealing at 57° for 30 sec, and an extension step at 72° for 35 sec. After a final extension step at 72° for 10 minutes, 10 µl of each cDNA product was stained with 1 µl of 1:10,000 dilution of SYBR Gold (Molecular Probes, Inc., Eugene, OR) and run on a 2% agarose gel. The size of the amplicon from the competitor RNA was 280 bp whereas the size for the amplicon from the endogenous pp32 and pp32r2 was 195bp and from pp32r1 was 185 bp. After gel electrophoresis, the gel was photographed under UV transillumination on the Strategene Eagle Eye system. The image was saved as a tiff file on a disk and analyzed for band intensities utilizing the TotalLab Software (Phoretix). Since incorporation of dye is dependent on size, the intensity of the smaller test band was multiplied by the ratio [competitor size/test amplicon size]. This was designated as the corrected test band intensity. Then, the ratio of the intensities of the competitor to the corrected test band intensity was obtained. A linear regression analysis was performed with the log copy number of the competitor on the X-axis and the log of the above ratio on the Y-axis. The point at which the regression line intersected the X-axis indicated an equal amount of starting endogenous template and exogenously added competitor. Figure 1 illustrates a representative calibration curve.

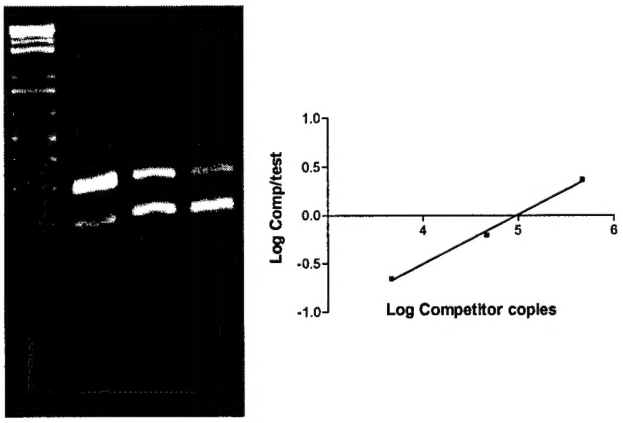

Figure 1. Representative Competitive RT-PCR Analysis of pp32. All lanes represent 20 ng of total RNA. A, 4.63 x 105 copies of competitor RNA; B, 4.63 x 104 copies; C, 4.63 x 103 copies. The graph shows the corresponding linear regression line (r2 = 0.9954, p = 0.0432).

Task 3. This approved task aims to adapt the molecular pathology assay for use archival tissue. Briefly, the task involves preparation of RNA from set of paraffin-embedded human prostatic adenocarcinomas, paired adjacent normal prostates, and control tissues. This is followed by amplification of pp32 mRNA by RT-PCR and analysis by the assay developed in Task 2, and validation of the assay by subcloning and sequencing of selected regions or entire inserts as indicated, using methods described for Task 1.

Progress: As with the assay outlined in the previous report, the assay outlined in Task 2 was specifically designed to be compatible with RNA fragments harvested from paraffin. The amplicons for pp32, pp32r1, and pp32r2 are each in the 300 to 350 bp range, which is generally compatible with the size of RNA fragments extractable from paraffin-embedded tissues. The recently developed assay noted above is now being applied to paraffin sections.

Task 4. This approved task seeks a preliminary determination of the clinical significance of pp32 molecular changes. This task requires assembly of paraffin blocks from previously studied population of prostatectomy specimens (1), preparation of RNA from paraffin sections, analysis of pp32 RNA by molecular assay developed under Tasks 2 and 3. The results will be selectively validated by selective subcloning and sequencing, as described in Task 1.

Progress: Work on this task remains scheduled to begin as soon as Task 3 is complete. Work will continue on this project beyond the funding period in order to accomplish the approved tasks despite the frustrating delays introduced by unanticipated and thorny problems in assay development. The clinical goals of the project were, and remain, valid.

KEY RESEARCH ACCOMPLISHMENTS

- Frustrating technical difficulties have finally been overcome through development of a reliable quantitative assay. The quantitative RT-PCR assay is now ready to apply to the other approved tasks. The quantitation overcomes difficulties with the large differences in expression levels among the pp32 gene family members (Task 2)

- The assay is applicable to paraffin sections and is now being validated (Task 3).

REPORTABLE OUTCOMES

None at present. Anticipate submission of manuscript describing assays shortly. Results of other approved tasks will follow.

CONCLUSIONS

Quantitative analysis of pp32 gene family members in human prostate cancer and benign tissues is now finally ready to be applied to clinical specimens as originally intended. The results are expected to be particularly informative since recent data, discussed in the Introduction, makes pp32 an interesting and potentially very important analyte. As a regulator of histone acetylation, transformation differentiation (1-4), and mRNA stability (5), it may yet prove to be both an important clinical determinant of the course of disease, as well as a therapeutic target.

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APPENDICES

Personnel Receiving Pay for Research Effort

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ShriHari S. Kadkol MD PhD	Co-Investigator
Moushira Mahmoud MBBS	Post-Doctoral Fellow
Bruce Huang	Technician

Publications & Abstracts during Project Period Including No-Cost Extension

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